Summary

Investigation of Zn^{2+}-dependent CD4 coreceptor and Lck tyrosine kinase assembly

The vast array of protein-protein interactions that occur in the cell is thought to be the basis of its functioning. Thus the full representation of protein-protein interactome is crucial and indispensable to understand cell molecular machinery at different levels. Among biomolecules present in the cell environment circulate metal ions with almost negligible size comparing to protein diameter but with the critical impact on both, structure and function of protein complexes. The metal ion that lays in the scope of the theis is Zn^{2+}, which influence on the human organism has been investigated for decades, however, its ability to bring together two proteins has just started to be revealed. The reason of a broad utility of Zn^{2+} in the protein complexes that play catalytic, structural, regulatory, and transporting roles is grounded in the Zn^{2+} capability to form bonds with different types of protein functional groups: cysteinyl, histydynyl, and carboxyl. Such versatility is reflected in a distinct Zn^{2+} coordinations and Zn^{2+} to protein stoichioetries resulting in a broad range of Zn^{2+} affinities, from micro- to picomolar. Presence of Zn^{2+} protein complexes that bind metal ion with such different strengths is one of the basis of Zn^{2+} being buffered in a cell. Proteins that are the main constituents of Zn^{2+} buffering system are Zn^{2+} transporters and a family of metallothioneins that are able to bind up to 7 Zn^{2+} with different affinities. As the result, in the cell there is a pool of Zn^{2+}_{free} that correspons to Zn^{2+} available for binding by proteins.

The heterodimeric Zn^{2+}-involved complex being the main subject of the theis is composed of 38-amino acid residue cytoplasmic tail of CD4 coreceptor and 29-amino acid residue Lck tyrosine kinase N-terminus. It is the first found Zn^{2+}-based interprotein complex that plays a crucial role in the immune system functioning. However, besides the high importance of its components on the T cell activation and T cell development processes, the impact of Zn^{2+} has been barely studied. The basis of the Zn(CD4)(Lck) complex called “zinc clasp” are two cysteinyl resiudes from each protein subunit that coordinate Zn^{2+} in the tetrahedral manner. Nevertheless, in the CD4 sequence one can find also other cysteinyl and histydynyl residues that may potentially contribute. In order to investigate the influence of sequential and structural parameters that reside in CD4 sequence on the CD4 and Lck assembly, the set of CD4 peptides of different lengths and mutations has been investigated using spectrophotometry, circular dichroism and competition studies. As the result, stabilities and stoichiometries of formed complexes have been determined pointing that the longest variant of CD4 is characterized with the highest affinity for Lck and also that the C^{397} and H^{399} are crucial for the Zn^{2+} and CD4 complexation. As long as C^{397} underlies reversible palmitoylation in the cell, one of the aims were to evaluate the influence of such modification on the zinc clasp assembly. Obtained by chemical synthesis palmitoylated CD4 were compared with its non-palmitoylated version in terms of Zn^{2+}-dependent complexation with and without Lck revealing that modification of C^{397} weakens the formation of the zinc clasp. Formation of Zn^{2+}-involved CD4 and Lck complexes were observed using fluorescently labeled protein domains in a buffering Zn^{2+} conditions. Application of inorganic Zn^{2+} buffers resulted in a competitive Zn^{2+} environment that led to the precise determination of Zn^{2+} complexes stability constants with $\log K^{12}$ for zinc clasp of 18.6. Moreover, it was shown that for dimeric Zn^{2+} complexes, the important parameter to obtain

functional level of an assembly is not only the concentation of Zn^{2+}, but also the concentration of protein subunits which stays in the contrary to Zn^{2+} complexes of other stoichiometries.

To track Zn^{2+} complexation by CD4 and Lck in a more reliable manner, the artificial model of a membrane was developed where immobilized protein domains were shown to assemble when Zn^{2+} was present, in the contrary to its absence. The results were obtained by the FLIM-FRET methodology that enabled to observe single liposomes that bore fluorescently labeled CD4 and Lck. Besides the application of an artificial membrane also the cellular studies were undertaken. At first, the wide range of intracellular Zn^{2+}_{free} concentration was obtained for Jurkat T cell line thanks to a different treatement of cellular media. After the acquiring of the cell line with stable CD4 expression ($CD4^+$) it has been shown that CD4 level on a membrane surface increases together with the increase of intrecellular Zn^{2+}_{free} concentration using FACS technique. $CD4^+$ cell line was then subjected to the transient transfection with fluorescently labeled Lck in order to observe FRET changes between CD4 and Lck. To illustrate influence of Zn^{2+} on the CD4 and Lck assembly, Lck mutants were approached that bore mutation of Zn^{2+}-binding residues. FACS-FRET data revealed that indeed, FRET levels of Lck mutants were significantly lower than the Lck that was capable of Zn^{2+} binding and also the T cell that were stimulated for activation were characterized by the higher level of the interaction than the non-stimulated ones.

In the course of the studies zinc clasp assembly was optimized towards development of the Zn^{2+}-based reversible toolset for protein dimerization. To maximize the specificity of heterodimer formation, CD4 mutant was chosen that were characterized with the highest stability towards Lck while the Zn^{2+}-based complexes of CD4 were the least stable. Efficiency of the heterodimerization and stability constants were determined with size exclusion chromatography, spectrophotometry, and fluorescence using the peptides and their derivative with fluorescent proteins. The utility of a CD4 sequence as a tag was shown in the native chemical ligation with small protein of bacterial origin. Obtained set of CD4-tagged proteins was then subjected to the analsyis with molecular baits that were based on the immobilized Lck peptide. It was shown that not only CD4-tagged targets themselves but also the overexpressed protein from *E.coli* lysate were reversibly catched according to the interaction with Zn^{2+}. Moreover, the specificity of a developed toolset with other metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}) were tested and the kinetic parameters were evaluated using biolayer interferometry technique. In order to investigate binding of Zn^{2+} to proteins and to determine the stability of Zn^{2+}-based complexes the frequently used approach is the application of metallochromophores. Two of the most commonly used, being PAR and Zincon, were deeply characterized regarding molar absorption coeffciencts and stability constants of their complexes with range of metal ions (Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, and Mn^{2+}) taking into account an inconsistent literature data available at that time. Obtained result helped not only to determine Zn^{2+} to proteins binding more reliably, but also provided comprehensive set of data ready to be used for scientists from analtical chemisty, environmental analytics, and biochemistry field of research.

Abbreviations: FLIM, fluorescence lifetime imaging; FRET, fluorescence resonance energy transfer; FACS, fluorescence-activated cell sorting; PAR, 4-(2-pirydylazo)resorcinol.